UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OCT - 4 1989

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Brodifacoum, Report of Apparent Human Self Poisoning, Further Information

TO: William Miller PM-16/5
Registration Division (H7505C)

FROM: Robert P. Zendzian Ph.D. 10/2/89
Senior Pharmacologist
SACB, HED (H7509C)

THROUGH: Albin Kocialski Ph.D. ef for ak 10/3/89
Head
Registration Standards and Special Review Section

Reto Engler Ph.D. 10/5/89
Chief
Science Analysis and Coordination Branch

On Thursday Sept 28, 1989 I received a telephone call from Dr. Mike Murphy of the University of Minnesota. Dr. Murphy has developed a very sensitive analytical method for brodifacoum in blood and tissue and wished to determine if I was aware of any need for his analytical capabilities. Dr. Murpy's address and phone numbers are;

Dr. Mike Murphy
1943 Carter Ave.
St. Paul, MN 55108

(612) 625-3197

During our conversation I mentioned that I had recently reviewed the medical records of an apparent human poisoning case and Dr. Murphy indicated that he has analyzed tissue from this case which indicated the presence of brodifacoum. Because of patient confidentiality, Dr. Murphy is unable to provide us directly with his data but we may expect to obtain it from the registrant in the same way we received the case history. Dr. Murphy has also analyzed blood samples from the recent local brodifacoum poisoning case of a 2-year old. He has promised to convey our interest in obtaining further information to the Doctor in charge of the case.

UNIVERSITY OF MINNESOTA
TWIN CITIES

Department of Veterinary Diagnostic Investigation
College of Veterinary Medicine
1943 Carter Avenue
St. Paul, Minnesota 55108
(612) 625-8787

September 28, 1989

Dr. Robert P. Zendzian
Health Effects Division, US EPA
Washington, DC 20460

Dear Dr. Zendzian,

I enjoyed our conversation September 28 regarding your interest in analytical methodologies to confirm exposure to second generation anticoagulant rodenticides. Three reprints of articles specifically dealing with this issue are enclosed for your convenience. During the past two years we have provided analytical evidence for the presence of brodifacoum using HPLC alone or in combination with GC/MS in eight human cases of exposure. Due to your interest in this area, I will contact the physicians involved in these cases and inform them of your interest. To my knowledge, three of these cases have been, or will be, submitted for publication by the physicians involved.

We have been actively pursuing the detection and treatment of brodifacoum toxicosis in the canine for the past six years. Copies of abstracts presented at the Society of Toxicology Meetings are additionally enclosed for your convenience. We have been able to confirm the presence of brodifacoum in the bile from one canine field case and have not detected its presence in the urine of field or experimental cases (human or canine). For these reasons, we are interested in considering the prevention of enterohepatic circulation as one mechanism of treatment.

Again, thank you for your time and interest.

Sincerely,

Mike Murphy DVM, PhD

Journal of Analytical Toxicology, Vol. 13, July/August 1989

The Determination of the Anticoagulant Rodenticide Brodifacoum in Blood Serum by Liquid Chromatography with Fluorescence Detection

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Abstract

A sensitive method utilizing reversed-phase liquid chromatography with fluorescence detection has been developed for the analysis of the anticoagulant rodenticide brodifacoum in blood serum. The serum proteins are precipitated with acetonitrile and the supernatant mixed with ethyl ether. The organic phase is separated, evaporated to dryness, and the residue subjected to chromatographic analysis. Extraction efficiencies of brodifacoum at concentrations of 20, 60, and 300 ng/mL were 82.9, 93.4, and 93.8%, respectively, with coefficients of variation (CVs) of 3.52, 4.07, and 3.68%, respectively. The intrarun precision (CV) without an internal standard at concentrations of 20, 60, and 300 ng/mL were 1.93, 4.89, and 1.51%, respectively, and 3.56, 5.94, and 3.66% with an internal standard. The interrun precision over the concentration range of 20–1000 ng/mL ranged from 1.88–6.22% without an internal standard and from 2.07–12.6% with an internal standard. Brodifacoum was measurable to at least the 1-ng/mL level.

Introduction

Brodifacoum (Figure 1) is a second generation anticoagulant rodenticide commonly used by professional pest control operators and in over-the-counter products. Inadvertent exposures in nontarget species, including dogs, cats, and humans, have been reported (1–4). In both humans and animals weeks of vitamin K1 therapy may be required to correct a coagulopathy due to brodifacoum exposure (2,5). A method to confirm absorption in cases of expected exposure, to evaluate exposure in coagulopathies of undetermined origin, and to determine the toxicokinetics of brodifacoum is needed.

Existing methods for brodifacoum detection are focused primarily on the analysis of baits or tissue residues and are generally too tedious and time consuming for the clinical setting. Tissue residue methods for brodifacoum have relied primarily on liquid chromatography with both normal and reversed-phase systems (6–10). Both fluorescence and UV detection have been used. In this report a reversed-phase liquid chromatographic system with fluorescence detection is presented for the analysis of brodifacoum in canine serum. Although developed on canine serum, the method has been successfully applied in human exposure cases and should prove valuable in the human toxicology setting as well.

Experimental

Materials. Brodifacoum and difenacoum were gifts from ICI Americas. Acetonitrile, methanol, and ammonium acetate were HPLC grade from Baker Chemical. Ethyl ether was anhydrous AR grade from Mallinckradt. Triethylamine was Aldrich Chemical gold label grade.

Apparatus. Chromatographic analysis was performed on a Hewlett-Packard 1090A chromatograph equipped with an automatic sampler, a ternary gradient pumping system, and a Shimadzu RF-530 fluorescence detector. Chromatographic peak areas were measured with a Hewlett-Packard model 3392 electronic integrator. The analytical column was a Beckman Instruments C_{18} Ultrasphere 250- × 4.6-mm column.

Chromatographic conditions. The mobile phase consisted of 84% methanol and 16% ammonium acetate/triethylamine buffer pumped at a flow rate of 1 mL/min. The methanol and buffer were mixed on line by the gradient pumping system. Separations were carried out at ambient temperature. The buffer was prepared by dissolving 3.85 g ammonium acetate, 2 mL

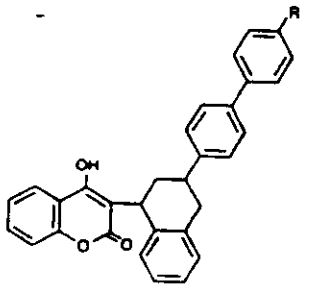

Figure 1. Chemical structure of brodifacoum (R = Br) and difenacoum (R = H).

acetic acid, and 2 mL triethylamine in 1 L distilled water. The fluorescence detector excitation wavelength was 285 nm and the emission wavelength was 390 nm.

Serum extraction. The difenacoum internal standard (100 μL, 2 mg/L) and 4 mL acetonitrile were added to 2 mL serum in a 15- × 125-mm screw cap culture tube. The tube was vortexed and centrifuged, and the supernatant was poured into a 25- × 150-mm screw cap culture tube. An additional 2 mL acetonitrile was added to the precipitate and vortexed. The acetonitrile was poured off and combined with the first extract. Approximately 12 mL of diethyl ether was added to the acetonitrile extract and mixed by inverting the tubes several times. The layers were allowed to separate and the bottom aqueous layer was removed with a disposable pasteur pipet and discarded. The ether layer was poured into a clean 25- × 150-mm culture tube and concentrated under a stream of nitrogen in a water bath at 55°C. When the volume was reduced to approximately 4 mL, the extract was transferred to a 12-mL conical centrifuge tube with a disposable pasteur pipet and evaporated to dryness under nitrogen at 55°C. The residue was reconstituted in 200 μL methanol. The tubes were briefly immersed in a sonic bath to aid in dissolution. The residue was transferred to an autosampler vial and 20 μL injected automatically onto the HPLC column.

Standard solutions and serum standards. A stock solution of brodifacoum (332 mg/L) was prepared by dissolving 8.3 mg brodifacoum in 25 mL of acetonitrile. Two working standard solutions of 2 mg/L and 20 mg/L were prepared by diluting the stock solution in methanol. Serum standards in the range of 10 to 1000 ng/mL were prepared by adding 10- to 100-μL quantities of the standard solutions to 2 mL of serum. The difenacoum internal standard stock solution (184 mg/L) was prepared by dissolving 4.6 mg in 25 mL acetonitrile. The working internal standard was prepared by diluting the stock solution to 2 mg/L in methanol. All standards were stored at −5°C.

Precision, accuracy, and linearity. Three sets of twelve 2-mL serum samples were spiked with brodifacoum to give final concentrations of 20, 60, and 300 ng/mL. Intrarun precision was measured using these samples. Six of the 12 samples were run with 100-μL internal standard (2 mg/L) and six without the addition of an internal standard. For linearity measurements, five sets of ten 2-mL serum samples were prepared. Each set was spiked with a brodifacoum concentration of 20–1000 ng/mL and stored at −25°C. The linearity of each set was determined on each of five consecutive days.

Results and Discussion

Chromatography. On the reversed-phase system used in this study brodifacoum is not resolved into its cis and trans isomers, but chromatographs as a single symmetrical peak (Figure 2). Brodifacoum is well resolved from background peaks found in canine serum. Other hydroxycoumarin rodenticides, namely warfarin and bromadiolone, elute at 2.7 min and 3.3 min, respectively, and do not pose an interference problem.

Extraction. A number of solvent systems were found to effectively extract brodifacoum from aqueous solutions. However, when applied to serum samples very little brodifacoum was extracted, indicating strong interactions with serum macromolecules. Precipitation of serum proteins with perchloric acid before solvent extraction did not significantly improve recoveries. Precipitation of the serum proteins with acetonitrile did result in satisfactory recovery of brodifacoum in the supernatant. Extraction efficiencies of brodifacoum at concentrations of 20, 60, and 300 ng/mL were 82.9% ± 3.52% (CV), 93.4% ± 4.07% (CV), and 93.8% ± 3.68% (CV), respectively, where n = 6. The addition of ethyl ether to the acetonitrile extract eliminates some of the water from the extract. This results in a more rapid evaporation of the extract and eliminates a large quantity of methanol insoluble material in the residue.

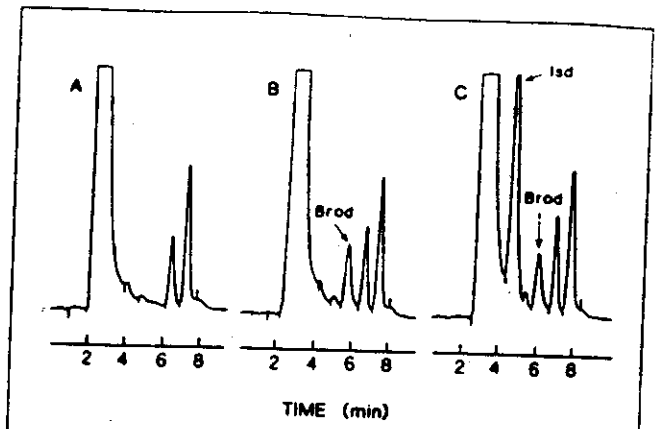

Figure 2. Chromatograms of (A) canine serum blank, (B) canine serum spiked with 20 ng/mL brodifacoum, and (C) canine serum spiked with 20 ng/mL brodifacoum and diphenacoum internal standard.

Internal standard. Diphenacoum (Figure 1) is an ideal candidate as an internal standard based on its physico-chemical properties. It is structurally similar to brodifacoum, flouresces with similar intensity, and is extracted with equal efficiency. However, its chromatographic behavior is less than ideal. Diphenacoum elutes from the C_{18} column relatively early and is not totally resolved from the void volume peak. It is therefore necessary to measure its area on a sloping base line. Attempts to move diphenicoum away from the solvent front by reducing the methanol composition of the mobile phase resulted in an unacceptably broad brodifacoum peak.

The method works well without the use of an internal standard. Use of difenacoum does not appear to significantly improve the precision or the linearity of the method; it is useful however to ensure that no gross errors have occured. Statistical results are presented for calculations based on analysis with and without the use of an internal standard.

Precision. The intrarun precision (CV) for brodifacoum at concentrations of 20, 60, and 300 ng/mL was 1.93%, 4.89%, and 1.51% (n = 6), respectively, with the use of an internal standard. The intrarun precision (CV) without the use of an internal standard at 20, 60, and 300 ng/mL was 3.56%, 5.94%, and 3.66% (n = 6), respectively. The interrun precision is shown in Table I for ten concentrations of brodifacoum in the range of 20–1000 ng/mL, run on five consecutive days. The coefficients of variation ranged from 1.88% to 6.22% without an internal standard and 2.07% to 12.6% with an internal standard.

Linearity. Straight lines were obtained over the concentration range of 20–1000 ng/mL when the concentration was plotted versus the peak area of brodifacoum or versus the ratio of the peak area of brodifacoum to the peak area of the internal standard. The linear regression parameters for calibration lines measured on five consecutive days are presented in Table II. The correlation coefficients ranged from .9924–1.000.

Table I. Interrun Precision Data

Concentration (ng/mL)	CV (%)* Without ISD**	With ISD***
20	5.84	3.42
40	5.30	2.07
60	4.63	10.9
80	4.85	4.19
100	5.34	7.94
200	6.22	5.71
400	1.88	3.60
600	1.92	3.58
800	3.10	5.96
1000	3.74	12.6

* Each value represents the mean of five analyses run on five consecutive days.
** Based on the measurement of brodifacoum peak area.
*** Based on the ratio of the internal standard and brodifacoum peak areas.

Table II. Linear Regression Parameters

Day	Intercept	Slope	Corr. Coef.
	Conc. vs. Peak Area		
1	29748	22102	.9973
2	−144155	22974	.9993
3	−62254	24167	1.000
4	−81970	23108	.9999
5	−94796	21656	.9999
	Conc. vs. Peak Area/ISD Ratio		
1	.1433	.0120	.9946
2	−.2380	.0154	.9924
3	−.0388	.0137	.9992
4	.0209	.0126	.9984
5	−.0222	.0134	.9999

Sensitivity. Brodifacoum can be readily measured at the 1-ng/mL level. At levels below 5 ng/mL the chromatographic injection volume should be increased to 50 μL. Also, the internal standard should be eliminated or reduced in concentration, since at these low levels the brodifacoum peak is obscured by the internal standard peak tail.

Application. The procedure described in this paper is successfully being used in a veterinary diagnostic setting to aid in the confirmation of suspected brodifacoum exposure in small animals. Figure 3 shows a chromatogram of a canine serum sample from a brodifacoum-poisoned dog. The animal died of pulmonary hemorrhage, congestion, and edema, and the owner had used a brodifacoum-containing rodenticide.

Although not developed specifically for use in human diagnostic toxicology, the method has been successfully applied to a number of human cases and should prove valuable in the human clinical setting. Figure 3 shows the chromatogram of human serum from a patient suspected of ingesting brodifacoum.

In cases which require further confirmation, the GC/MS procedure of Ray et al. (11) has been used in conjunction with HPLC. HPLC positive samples are subjected to chromic acid degradation. Both brodifacoum and bromadiolone are degraded to bromobenzoic acid which is then analyzed by GC/MS.

In summary, we have presented a method for the measurement of brodifacoum in blood serum which should be of value for the diagnosis of brodifacoum poisoning, as well as for toxicokinetic studies of the compound.

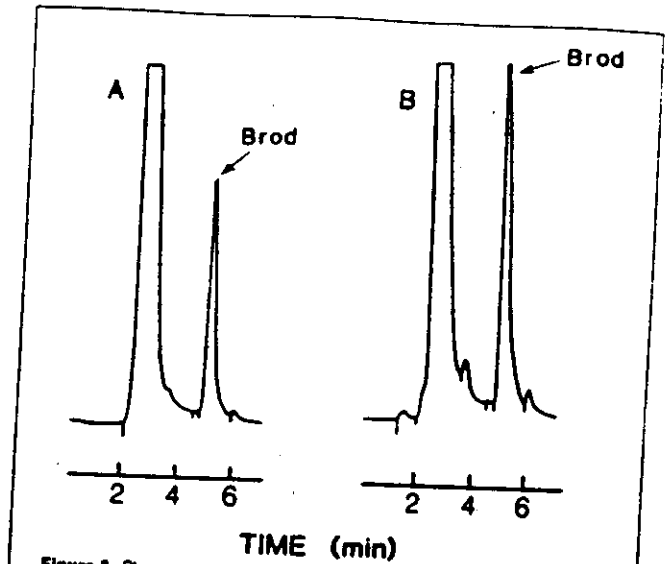

Figure 3. Chromatograms of (A) canine serum containing 50.0 ng/mL from a dog poisoned with brodifacoum and (B) of human serum containing 97.5 ng/mL brodifacoum from a poisoning victim.

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Determination of brodifacoum and bromadiolone residues in rodent and canine liver

Allen C. Ray, PhD; Michael J. Murphy, DVM, PhD; Michael D. DuVall, DVM, MS; John C. Reagor, PhD

SUMMARY

A method to determine residue concentrations of anticoagulant rodenticides, brodifacoum (BF) and bromadiolone (BD) in liver was developed, using gas chromatography/mass spectrometry. Nine dogs were given 1.1 mg of BF/kg of body weight, PO, in polyethylene glycol 400, one time. Rats were fed BF or BD (via commercial baits) in amounts from 0.28 to 11.25 mg/kg over 1- to 4-day periods. Fresh liver samples were collected at necropsy from all rats and 3 dogs, ground with Na_2SO_4, and extracted with $CHCl_3$:MeOH (9:1). After evaporation and silica cartridge purification were performed, residues were oxidized with a 0.16*M* chromic acid solution, and an oxidation product (4-bromobenzoic acid) was partitioned into $CHCl_3$. The methylated derivative (port derivatization with trimethylanilinium hydroxide) was assayed, using gas chromatography/mass spectrometry. Bromadiolone was detected in livers from rats given > 6 mg of BD/kg of body weight, but not in livers of rats given 1.25 mg of BD/kg. In contrast, BF was detected (with one exception) in livers from dogs (given 1.1 mg of BF/kg) and from rats given high (11.25 mg of BF/kg) and low (0.28 mg of BF/kg) doses. This protocol, which does not differentiate between BF and BD because of the formation of a common product after chromic acid oxidation, was used to diagnose anticoagulant toxicosis in 3 dogs, 1 human being and 1 llama naturally poisoned.

The evolution of warfarin-resistant rats has resulted in the introduction of more effective anticoagulant rodenticides, specifically brodifacoum (BF)[a] and bromadiolone (BD).[b] The increased availability of these second-generation products has resulted in a concurrent increase in the number of exposures and poisonings in nontarget species. In 1986, the National Animal Poison Control Center reported that inquiries regarding rodenticides had increased to 17% of calls, and these products were the most frequently involved class of agents cited. Over 72% of calls concerning anticoagulant-related toxicoses involved second-generation compounds, the greatest increase being calls about BF.[1]

The new anticoagulants are similar in action to warfarin and related compounds, ie, they inhibit vitamin K epoxide reductase,[2] but generally are eliminated more slowly than is warfarin.[3] Dogs and rabbits ingesting BF may experience coagulopathy for several weeks.[4,5] The LD_{50} values for BF and BD in rats and dogs range from 0.2 to 3.6 mg/kg of body weight.[6-8]

Confirmation of poisoning as a result of these compounds is a considerable diagnostic problem, because other causes of coagulopathy (ehrlichiosis, infectious canine hepatitis, disseminated intravascular coagulopathy, autoimmune thrombocytopenia, aflatoxicosis, heat stroke, and inherited clotting-factor deficiencies) are sometimes encountered. Current methods use high-pressure liquid chromatography (HPLC)[9-13] and gas chromatography (GC)[14] to determine BF and diphenadione, respectively, in tissues, but these methods, although sensitive, lack the selectivity necessary for unequivocal confirmation, unless multiple chromatographic modes and derivative spectra are used.[15] The purpose of the study reported here was to develop a method to detect residue concentrations of BF and BD in tissue, using GC/mass spectrometry (MS) after oxidation of extraction residues by chromic acid.

Materials and Methods

Animals—Eight male and 4 female adult Sprague-Dawley rats weighing 400 to 450 g were used. Rats were maintained in polycarbonate cages and had free access to water and, except for designated periods of bait exposure, feed. Nine dogs of mixed breeds and of both sexes, weighing 11 to 20 kg, were kept in 1.8- × 1.8-m runs or in metabolism cages in heated rooms and were given free access to food and water. Hepatic and renal tissue from 3 dogs, 1 person, and 1 llama naturally poisoned were submitted to our laboratory for diagnostic evaluation.

Dosing regimen—To simulate conditions of natural poisonings, rats were fed commercial bait[c] containing 50 μg of BF/g of bait in measured amounts after not being fed for 24 hours. Single doses of bait corresponding to 0.28 mg of BF/kg of body weight (LD_{50} dose)[8] were weighed and fed to 3 rats in individual cages. The bait was consumed within 6 hours by each rat. Multiple doses of 30 g each of bait were fed to 4 rats in individual cages for 24 hours over 5 days. At the end of each 24 hours, the remaining feed was weighed to determine the amount ingested by each rat (total dose, 7.5 to 11.25 mg of BF/kg).

Dogs were given one dose of 1.1 mg of technical grade BF in polyethylene glycol 400/kg, PO, via gastric tube. Nine dogs were

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From the Texas Veterinary Medical Diagnostic Laboratory, PO Drawer 3040, College Station, TX 77841.
Supported by grants from the AVMA Foundation and the Alpo Veterinary Grant Program.

[a] Brodifacoum, ICI Americas Inc, Wilmington, Del.
[b] Bromadiolone, Chempar Chemical Co, Schenectady, NY.
[c] Talon G, ICI Americas Inc, Wilmington, Del.

itored closely (every 2 to 4 hours) for the appearance of clinical signs of toxicosis. Clotting values (prothrombin times, activated clotting times) also were closely monitored, and when these assays indicated coagulopathy, treatment with vitamin K_1 was instigated in all 9 dogs. Three male dogs also were given 2 blood transfusions each, but did not respond and died despite efforts to keep them alive. Livers from these 3 dogs were used in the method evaluation. Other dogs recovered without complications after vitamin K_1 treatment.

Under the same conditions as the BF study, rats were fed commercial bait[d] containing 50 μg of BD/g of bait in measured quantities ranging from LD_{50}[7] amounts (1.25 mg of BD/kg, single dose, 3 rats) to 6.75 mg of BD/kg and 10.6 mg of BD/kg (multiple doses of 30 g each/24 hours for 5 days, 2 rats).

In both studies, rats were monitored every 2 to 6 hours for the appearance of clinical signs of toxicosis (eg, lethargy, hematomas, epistaxis), at which time rats were euthanatized in CO_2 chambers. Rats that survived for 7 days and had no clinical signs of toxicosis also were euthanatized. Five rats that had no clinical signs of toxicosis were found dead on days 5 to 7. Livers from all rats were collected at necropsy.

Extraction technique—Tissue (liver or kidney, 10 to 12 g) was ground with 20 g of Na_2SO_4 in a mortar and pestle and was extracted with 100 ml of $CHCl_3$:MeOH (9:1) on a rotary shaker (30 minutes). After the residue was evaporated to dryness in a fume hood, it was dissolved in 1 ml of $CHCl_3$ and was loaded onto a silica cartridge,[e] and anticoagulants were eluted with 5 ml of reagent-grade $CHCl_3$; (containing 0.75% ethanol as preservative). The eluate was transferred to a large glass tube, and the $CHCl_3$ was evaporated. The residue was oxidized for 1 hour at 90 C with 20 ml of chromic acid solution (0.16*M* in acetic acid).[14] Distilled H_2O (20 ml) was added, and oxidation products were extracted with $CHCl_3$ (2×, 40 ml each). Pooled organic fractions were dried by filtering through Na_2SO_4 and were evaporated to dryness. After residues were dissolved in 0.5 to 1 ml of $CHCl_3$ and were transferred to calibrated tubes, 200 μl of trimethylanilinium hydroxide (TMAH)[f] was added just before analysis.

To determine the fate of the coumarin nucleus during oxidation and whether brominated environmental contaminants produced interfering substances, chemicals (eg, 3 mg of 4-hydroxycoumarin and 8 mg of polybrominated biphenyl [PBB][g]) to be evaluated were transferred to large glass tubes. Oxidation, extraction, and derivatization were performed as described for tissue samples.

GC/MS analysis—Methyl-4-bromobenzoate (MBB) was analyzed by GC/MS,[h] using a 1.83-m × 2-mm (ID) glass column packed with 3% stationary phase[i] on 100- to 120-mesh solid support.[j] Operating conditions were: injection port, 266 C; temperature program, 120 C to 270 C at 10°/min; solvent delay, 2 minutes; ion monitored, 183 or 185 mass/charge (m/z); injection volume, 1 to 5 μl; flow rate, 30 ml of zero-grade helium/min.

HPLC analysis—To measure quantitatively the conversion of BF and BD to oxidation products in yield and kinetic studies, 4-bromobenzoic acid (BBA)[k] was analyzed by HPLC[l] with a dual wavelength detector, dual pen recorder, and a reverse-phase column[m] (5 μm; 25 cm × 4.6 mm). The solvent system was 1.5% acetic acid (pH 4.5):acetonitrile HPLC grade[n] (1:1). Operating conditions were: flow rate, 2 ml/min; chart speed, 1.25 cm/min; UV detection, 254 nm; sensitivity, 0.2 absorbance units full-scale; injection volume, 1 to 5 μl.

Spiking techniques—Liver specimens from 3 dogs dying of natural causes and submitted to the Diagnostic Laboratory were used in spiking experiments. After livers were ground with Na_2SO_4, they were transferred to 500-ml Erlenmeyer flasks, and BF and BD were added to produce final concentrations of 2 μg and 20 μg of BF/g of liver and 5 μg of BD/g from standard solutions in $CHCl_3$ (2 μg/μl). After samples were shaken for 30 minutes with the extracting solvent mixture, they were oxidized, extracted, and assayed, as described previously.

Kinetics and yields of BBA formation—Brodifacoum (2 mg) or BD (2 mg; both from standard solutions containing 1 mg/ml in $CHCl_3$) was transferred to oxidation tubes, and the solvent was evaporated. Chromic acid solution (10 ml) was added, and solutions were heated at 90 C for 15 minutes to 2 hours. Extraction was as described previously. Residues were dissolved in 1 ml of MeOH before HPLC analysis. Identification of products was accomplished by GC/MS analysis.

Calculations—Standard curves for BBA or MBB were established by measuring peak heights or areas from HPLC or GC/MS chromatograms. Yields were calculated by the following equation:

$$\{[\text{mg of BBA/ml} \times (V \times CF)]/W\} \times 100 = \text{percentage of yield of BF or BD}$$

where V = sample volume in milliliters, CF = conversion factors (2.61 for BF, 2.63 for BD), and W = weight of BF or BD in milligrams.

Concentration in liver was calculated as follows:

$$[\mu\text{g of MBB (as BBA)}/\mu\text{l} \times (V \times CF)]/W = \mu\text{g of BF or BD/g}$$

where V = sample volume in microliters and W = weight of liver in grams.

$$\overline{D} = \Sigma (Xi - \overline{X})/n$$

where Xi = individual reading, $\overline{X}$ = mean, and n = number of replications.

Results

Identification of methylated products of chromic acid oxidation of BF and BD was accomplished by GC/MS analysis (Fig 1). The primary oxidation product, BBA, was identified after TMAH methylation by matching its retention time (4.8 minutes) and mass spectrum (Table 1) with that of an authentic standard. The TMAH methylation reaction is a flash alkylation occurring in injection ports heated above 260 C. Minor oxidation products include (1,1'biphenyl)-4-Br,4'-carboxylic acid (more prevalent at shorter oxidation times), 1,4-benzenedicarboxylic acid, and 1,2-benzenedicarboxylic acid (from BF only). Oxidation fragments of the coumarin nucleus from BF or BD were not recognized. Apparently, this structural unit does not survive the chromic acid treatment, because fragments of standard 4-hydroxycoumarin could not be detected after oxidation and extraction. To determine whether ubiquitous PBB mixtures contained a monobrominated analog that would yield BBA on oxidation and possibly lead to

[d] Just-One-Bite, Farnam Co Inc, Omaha, Neb.
[e] Sep-Pak, Waters Associates, Milford, Mass.
[f] Regis Chemical Co, Morton Grove, Ill.
[g] Firemaster, Michigan Chemical Co, St Louis, Mich.
[h] 5992B, Hewlett-Packard Co, Palo Alto, Calif.
[i] OV 101, Ohio Valley Specialty Chemical Inc, Marietta, Ohio.
[j] Chromosorb W-HP, Johns-Manville Co, Denver, Colo.
[k] Aldrich Chemical Co, Milwaukee, Wis.
[l] ALC/GPC 204, Waters Associates, Milford, Mass.
[m] Supelcosil C-18 DB, Supelco Inc, Bellefonte, Pa.
[n] Fisher Scientific Co, Fair Lawn, NJ.

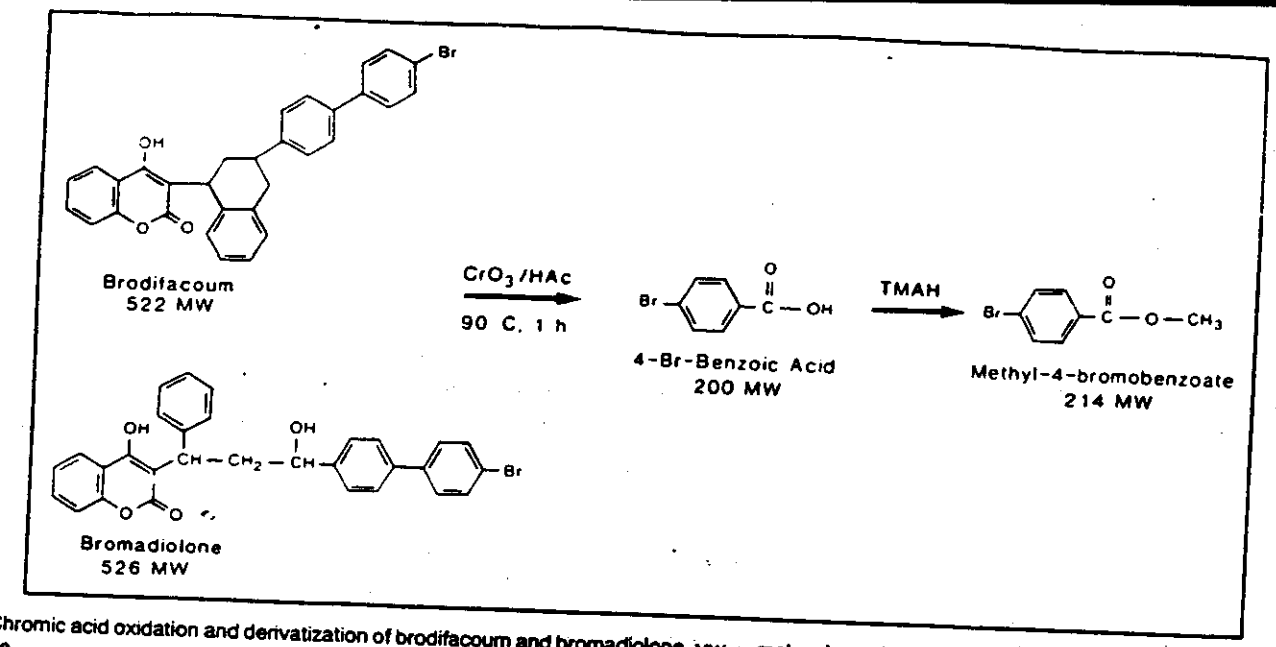

Fig 1—Chromic acid oxidation and derivatization of brodifacoum and bromadiolone. MW = molecular weight; HAc = acetic acid; TMAH = trimethylanilinium hydroxide.

TABLE 1—Mass spectrum of methyl-4-bromobenzoate

Mass (m/z)	Abundance (%)
50	46
51	7
59	4
63	5
73	9
74	40
75	65
76	57
77	9
91	2
92	2
103	2
104	9
105	2
119	2
135	10
155	44
156	5
157	41
158	4
183	100
184	10
185	98
186	9
214	38
215	7
216	38
217	3

m/z = mass/charge.

erroneous results, the PBB preparation was subjected to the assay procedure; BBA was not detected.

Conversions of standard BF and BD to BBA after oxidation were determined by HPLC (retention time of BBA, 2 minutes). Yields of BBA were 72.2% from BF (3 replicates; $\bar{D}$, ± 5.8%) and 55.3% from BD (3 replicates; $\bar{D}$, ± 2.6%).

Kinetic studies with BD revealed that BBA formation was essentially complete at 15 minutes and had not diminished at 2 hours. The 1-hour incubation time for liver was chosen as a means of removing excess coextractives.

Absolute recovery values of BF from liver samples spiked with 20 μg of BF/g of tissue (4 replicates) and 2 μg of BF/g of tissue (4 replicates) were 47% ($\bar{D}$, ± 3.6%) and 36.9% ($\bar{D}$, ± 2.4%), respectively. However, if corrected for the actual yield of BBA from BF, as established previously, these numbers increased to 66% ($\bar{D}$, ± 5.1%) for the 20 μg/g spike and to 51.8% ($\bar{D}$, ± 3.3%) for the 2 μg/g spike. Consequently, recoveries of BD from liver spiked with 5 μg of BD/g of tissue (3 replicates) were 49.8% ($\bar{D}$, ± 5.6%) as absolute values and 90.1% ($\bar{D}$, ± 10.1%) if corrected for the actual yield of BBA from BD. Nonspiked liver extracts were free of coeluting interferences.

Characteristic fragments in the mass spectrum of MBB (Table 1) were dominated by the presence of ^{79}Br and ^{81}Br isotopic peaks, which served as naturally occurring markers and produced peaks of approximately equal intensity which were separated by 2 mass units. These doublets at 155 and 157 m/z, 183 and 185 m/z, and 214 and 216 m/z for peaks at 4.8 minutes can be considered as unequivocal evidence for the presence of MBB. Unmethylated BBA will not elute from the GC column. Before injection, BBA can be methylated by another choice of alkylating reagents; MBB is formed by reaction with dimethyl formamide-dimethylacetal at 110 C for 1 hour (as determined by HPLC and confirmed by GC/MS). Derivatization with TMAH was chosen for the present study, because the reaction is instantaneous.

Although the progression of anticoagulant poisoning in animals is well documented, rats given low (LD_{50}) doses of BD and BF (Table 2) had no outward signs of clinical illness. At necropsy, lesions were minimal, but 2 rats given BF had small amounts of blood in the thoracic cavity and 1 rat given BD had pulmonary congestion. In rats given higher doses of BF or BD, lesions were more pronounced and were confined to pulmonary and abdominal hemorrhages. Bait intake decreased in these rats by the 4th day. Signs of clinical toxicosis were not apparent until at least the 5th day. Lesions in the 3 dogs given BF were confined to pulmonary congestion and hemorrhage. All lesions were consistent with those of earlier reports.[6-8]

dogs

Animal No.	Sex	Total dose (mg)	Dosage (mg/kg)	Survival time (days)	Rodenticide concentration* (μg/g)
BRODIFACOUM					
Rats					
1	F	4.25	10.6	6‡	6.5
2	M	3.0	7.5	5	8.1
3	F	4.0	10.0	5	21.0
4	F	4.5	11.25	7	0.9
5	M	0.13	0.28†	7‡	2.2
6	M	0.13	0.28†	7‡	0.4
7	M	0.13	0.28†	7‡	< 0.06
Dogs					
1	M	18.0	1.1†	7	0.08
2	M	31.0	1.1†	5	0.4
3	M	22.5	1.1†	11	0.3
BROMADIOLONE					
Rats					
1	F	4.25	10.6	6	2.2
2	M	2.7	6.75	6	2.0
3	M	0.51	1.25†	5‡	< 0.06
4	M	0.51	1.25†	5‡	< 0.06
5	M	0.51	1.25†	5‡	< 0.06

* Not corrected for recovery; < 0.06 = none detected. † Single oral dose. ‡ Euthanatized.

TABLE 3—Brodifacoum (BF) residues in tissue from species poisoned in the field

Species	Organ	Calculated as BF* (μg/g)
Llama	Liver	1.1
Dog	Liver	0.95
Dog	Liver	0.33†
Dog	Kidney	0.010†
	Liver	< 0.006†
Human being	Kidney	0.059†
	Liver	0.057†

* Not corrected for recovery; < 0.006 = none detected. † Determined by capillary gas chromatography/mass spectrometry.

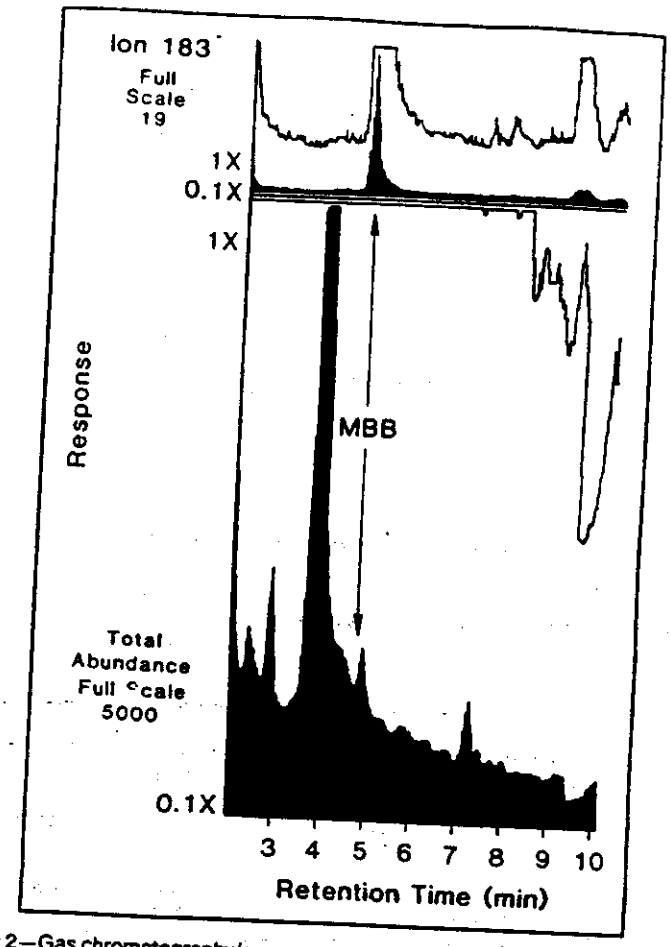

Fig 2—Gas chromatography/mass spectrometry chromatogram for analysis of methyl-4-bromobenzoate (MBB) from liver extract of rat 3 given 4 mg of brodifacoum. Ion 183 m/z = single ion monitoring; abundance = total or single ion counts. MW = molecular weight; m/z = mass/charge.

Analyses of hepatic tissue from these dogs and rats (Table 2) and from hepatic and renal tissue from naturally poisoned species (dogs, human beings, and llama, Table 3) for MBB (expressed as BF or BD) indicated that residues from BD were usually less than were residues from BF, and that kidney may be as suitable a sample for analysis as liver. None of these values was corrected for recovery. A sample chromatogram from analyses of these liver extracts (Fig 2) revealed the presence of selected ion (183 m/z) and total ion peaks. Characteristic mass spectral doublets were detected and offer confirmation for the presence of MBB. However, at low concentrations, the detection of mass peaks 183 m/z and 185 m/z at the proper retention time is considered to be sufficient evidence for MBB, because extracts of livers from dogs dying of natural causes were devoid of these peaks in the chromatographic region of interest. The minimal amount of BF or BD that can be detected in liver is approximately 0.06 μg/g.

Discussion

Analysis by GC/MS after chromic acid oxidation of liver extracts does not differentiate between BF and BD because they yield the same product, but conversely offers the advantage of screening with one protocol for the 2 most commonly used rodenticides. This technique also offers comparable sensitivity and much-improved selectivity as contrasted with existing HPLC methods. In the present study, BD was less persistent in liver and may be eliminated more rapidly than is BF. This observation is consistent with the comparative toxicities of the 2 compounds.[6-8] As expected, higher doses of BF or BD resulted in more severe lesions and clinical signs of poisoning and, generally, higher concentrations in the liver.

Trials in rats were conducted to provide authentic samples for method evaluation and were designed to simulate natural poisoning conditions. In addition to spiking experiments, dosing of animals was necessary to address such variables as metabolism, binding, and disposition of the toxicant. In dogs, uncharacterized factors apparently influence the toxicity of BF, as evidenced by the wide range of LD_{50} values.[6,7] The selected dose was in the lower end of that reported spectrum. Because monitoring and therapy were aggressive, unknown predisposing factors may have contributed to the demise of the 3 dogs.

Recoveries and conversions for these compounds are rather low, but precise and adequate for diagnostic and residue work. Sensitivity is certainly adequate, but could be improved by use of an electron-capture detector, with a concurrent loss of selectivity. Preliminary work with capillary GC/MS is promising, with sensitivity increased approximately tenfold. This method also has been adapted to bait and blood samples. Several unexplained coagulopathies in human beings have been confirmed as anticoagulant toxicoses, using GC/MS.[16]

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J Vet Diagn Invest 1:66–68 (1989)

Case studies on second-generation anticoagulant rodenticide toxicities in nontarget species

Michael D. DuVall, Michael J. Murphy, Allen C. Ray, John C. Reagor

Abstract. Specimens from 10 cases of second-generation anticoagulant rodenticide poisoning in dogs and cats were submitted to the Texas Veterinary Medical Diagnostic Laboratory during 1986 and 1987. The clinical signs most frequently observed were lethargy, dyspnea, and ventral hematomas; common necropsy findings included hemoperitoneum, hemothorax, and pulmonary hemorrhage. In the instances when histopathological examination of the tissue was done, it supported a diagnosis of coagulopathy. The presence of anticoagulants in serum or liver was confirmed by high pressure liquid chromatography, gas chromatography/mass spectrometry, or a combination of the two. Five cases of brodifacoum poisoning, 2 of bromadiolone, and 3 of diphacinone toxicity were verified. Concentrations of these rodenticides ranged from approximately 0.001 to 12 ppm.

The evolution of the warfarin-resistant rat has resulted in the development and marketing of several more effective rodenticides. These new compounds, collectively referred to as second-generation anticoagulant rodenticides, include brodifacoum (BF), bromadiolone (BD), difenacoum, diphacinone (DC), and chlorophacinone. This group of anticoagulants has greater efficacy in warfarin-resistant rats because of increased potency and prolonged duration of action. The effectiveness of these rodenticides has led to increased usage in bait formulations available commercially, which has created a significant problem of anticoagulant toxicosis in nontarget species, principally dogs and cats.[5]

Although some cases of suspected DC toxicity have been reported,[1] little information is available concerning analytical confirmation of rodenticide poisoning. The purpose of this report is to summarize the clinical signs, treatment, lesions, and analytical results of cases of second-generation anticoagulant rodenticide poisoning submitted to the Texas Veterinary Medical Diagnostic Laboratory during 1986 and 1987.

Materials and methods

Case histories. Nine canine (6 male and 3 female) and 1 feline (male) poisoning cases were studied (Table 1). Five of the dogs were found dead after appearing healthy hours before (2 had known exposure to DC and 1 to BD). The remaining 4 dogs and cat were presented exhibiting a variety of clinical signs. Three of these dogs subsequently died, whereas 1 dog and the cat recovered following vitamin K_1 therapy (both of these animals were known to have ingested anticoagulant bait). One dog did not respond to vitamin K_1 therapy. Clinical signs included lethargy, hematuria, dyspnea, altered gait, and ventral hematomas.

Also included in this report is the BF concentration from a case of human anticoagulant toxicity. The sampling was prompted by coagulopathies of unknown origin. In this instance, the gas chromatography/mass spectrometry (GC/MS) method designed for tissue was adapted to serum as an alternate and unequivocal means of confirmation.

Pathology procedures. All organs were examined grossly, and representative samples were fixed in 10% buffered neutral formalin. Fixed tissues were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

Analytical procedures. Diphacinone (DC) content of liver was determined by adapting published methods[2] to capillary or packed-column GC/MS for the analysis of benzophenone, the product of chromic acid oxidation. Ions 105 and 182 m/z were selectively monitored by GC/MS.

Brodifacoum (BF) concentrations in serum were determined using high pressure liquid chromatography.[4] Brodifacoum (BF) and BD concentrations in liver were determined utilizing chromic acid oxidation methods[6] with packed-column or capillary GC/MS analysis for the common derivation product, methyl-4-bromobenzoate (MBB). Ions 214 and 216 m/z were selectively monitored. Because the derivation of BF and BD yields the same product, it was necessary to establish a history of exposure in order to differentiate between the two. If exposure could not be established, the result was expressed as BF, the product more available commercially.

Results

Pathological findings. The most frequently observed postmortem findings were hemoperitoneum, hemothorax, and pulmonary hemorrhage. Specifically, lesions included hemopericardium, suffusive suben-

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Table 1. Histories and lesions of anticoagulant-poisoned animals.

Case no.	Species	History	Necropsy findings	Histopathology findings	Comments
1	Canine (male)	Presented with hematuria, weakness; unresponsive to vitamin K_1 therapy; died	Diffuse hemorrhages around base of heart, kidney, and bladder, and within omentum; reddened bladder mucosa; subendocardial hemorrhage	Purulent cystitis; hemorrhage of heart, mesentery, and perirenal fat	No known exposure
2	Canine (male)	Previously normal; found dead	Epistaxis; pale mucous membranes; petechiation of serous surface of lungs; congested intestine; hemoperitoneum	No significant lesions	Violent struggle prior to death suggested
3	Canine (male)	Found dead	Hemopericardium; free blood at tracheal bifurcation and in digestive tract; hematuria	Not done	Known exposure to diphacinone bait
4	Canine (male)	Found dead	Hemothorax; clot in anterior mediastinum	Acute centrolobular congestion and vacuolar swelling of heparocytes	Known exposure to diphacinone bait
5	Canine (female)	Found dead; bleeding from mouth	Hemothorax; hemoperitoneum; hemorrhagic lungs and free blood in intestinal lumen; melena	Not done	
6	Canine (male)	Presented for fire ant bites; died during night	Hemoperitoneum; congested lungs; pale liver	Congestion and terminal edema of lungs	
7	Canine (female)	Lethargy; labored breathing; ventral hematomas; responded to vitamin K_1 therapy			Ingested brodifacoum bait
8	Feline (male)	Lethargy; responded to vitamin K_1 therapy			Ingested brodifacoum bait
9	Canine (male)	Bleeding from mouth; seizure; presented dead	Hemothorax; unclotted blood in cardiac chambers	PMA; pulmonary congestion and edema	Known exposure to bromadiolone bait
10	Canine (female)	Presented comatose; prolonged clotting time; died shortly after	Hemothorax; hematomas	Acute hemorrhage (subcutaneous and around trachea)	Known exposure to bromadiolone bait

docardial hemorrhage, free blood in the lumen of the small intestine, atelectasis with free blood at the tracheal bifurcation, petechiations on the serosal surfaces of the lungs, and hemorrhagic bladder mucosa. Although histopathology was consistent with the gross findings and indicative of a coagulopathy, it was not diagnostic for anticoagulant intoxication. Lesions included diffuse interstitial cardiac hemorrhage and auricular carditis, acute centrolobular congestion and vacuolar swelling of hepatocytes, massive pulmonary edema and congestion, hemorrhages in the mucosa of the urinary tract and the serosa of the bladder wall, and hemoglobinuric nephrosis.

Analytical results. Results of analyses of sera and liver specimens for second-generation anticoagulant rodenticides are shown in Table 2. Generally, very low concentrations of BF were detected in sera, while higher levels were found in liver. Recoveries of BF and BD from spiked liver samples were in the 60–70% range, accounting for the 55–70% yields of MBB from these compounds (unpublished data).

Analysis of human serum for confirmation of ingestion of BF yielded a rather high concentration of BF as compared to those found in animals, suggesting a massive and/or recent dose.

Discussion

Second-generation anticoagulant rodenticide poisoning in nontarget species is a problem of increasing concern to both clinicians and diagnosticians. Vitamin K_1 therapy is effective, but must be continued over a 4–6-week period to avoid a recurrence of bleeding.[3] Initial diagnosis is difficult, because other causes of coagulopathy (ehrlichiosis, infectious canine hepatitis,

Table 2. Results of anticoagulant assays of specimens from poisoned animals.

Case no.	Sample	Method of analysis	Result (conc.)
1	Serum	HPLC	ND* (brodifacoum)
	Liver	GC/MS	Brodifacoum (2.0 ppm)
2	Serum	HPLC	Brodifacoum (0.001 ppm)
	Liver	GC/MS	Brodifacoum (1.2 ppm)
3	Liver	GC/MS	Diphacinone (0.21 ppm)
4	Liver	GC/MS	Diphacinone (0.15 ppm)
5	Liver	GC/MS	Brodifacoum (12.7 ppm)
6	Liver	GC/MS	Diphacinone (0.11 ppm)
7	Serum	HPLC	Brodifacoum (0.002 ppm)†
8	Serum	HPLC	Brodifacoum (0.009 ppm)†
9	Liver	GC/MS	Bromadiolone (0.16 ppm)
10	Liver	GC/MS	Bromadiolone (0.039 ppm)
Human (female)	Serum	HPLC, GC/MS	Brodifacoum (1.5 ppm)

* ND = none detected.

† Initial concentrations. The serum BF concentrations were monitored until levels dropped below 0.5 ppb.

disseminated intravascular coagulopathy, heat stroke, aflatoxicosis, inherited clotting factor deficiencies, etc.) must be considered. Clinical chemistry assays demonstrating clotting deficiencies are useful, but either a history of exposure or analytical confirmation is necessary to verify diagnosis. Presenting signs, gross lesions, and microscopic lesions are supportive and predictable, but not diagnostic. Screening methods are available for baits and feeds,[7] but no single method exists for the multiresidue determination of anticoagulants in tissues. At present, an analyst must rely on multiple and complementary methods to assay for low concentrations of various rodenticides.

Although concentrations are low, BF persists in sera for 2–3 weeks, and excretion is represented by a 2-compartment open model.[4] However, persistence of BF and other second-generation anticoagulants in liver has not been defined, and longer periods may be required for these compounds to be eliminated completely.

Second-generation anticoagulant rodenticides are both toxic and hazardous to nontarget species, and accurate diagnosis is essential for favorable prognosis and prevention of additional poisonings. As analytical support is expensive, time-consuming, and not widely available, often a detailed clinical history and workup are the only criteria on which to base the diagnosis. There is a need for selective and sensitive multiresidue methods to diagnose second-generation anticoagulant poisonings.

Two of the 10 cases in this report responded successfully to vitamin K_1 therapy. In both instances, the serum BF concentrations were monitored until the concentrations dropped below 0.5 ppb, at which time vitamin K_1 therapy was discontinued. These cases demonstrate the value of utilizing serum concentrations in evaluating treatment regimens. Unfortunately, only BF can be detected in sera at this time, necessitating that methods for detecting the other second-generation anticoagulants be developed.

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349 EFFECT OF CHRONIC EXPOSURE TO LEAD ON IN VITRO CONTRACTILE RESPONSE OF THE RAT FORESTOMACH. C.T. Walsh, E.B. Ryden and K.M. Harnett. Dept. of Pharmacology, Boston University Medical Center, Boston, MA. Sponsor: J.K. Marquis.

Previous studies have demonstrated that chronic exposure to lead in rats slows gastric emptying. The contractile response of the forestomach from lead-treated rats was examined in vitro. Male Wistar rats were fed 4% lead acetate in their diet (NIH-07); controls were pair-fed. After 7 weeks, blood was collected (180±26ug lead/dl) and the forestomach dissected. Tissue was suspended in a physiologic saline which for lead-treated rats contained 1.2×10^{-5}M lead. Chronic lead exposure had no effect on the maximum tonic contraction induced by KCl, methacholine or serotonin. Lead-treated tissue showed enhanced sensitivity to methacholine (1.6×10^{-7}M), as has been shown previously. Physostigmine-induced increase in tension was also significantly greater in tissue from lead-treated rats. Electric field stimulation (1Hz, 1msec, 100V) produced a contraction attributable to postganglionic acetylcholine release. This response was unaltered in lead-treated tissue. These results indicate that lead intoxication did not impair the contractile apparatus of the forestomach smooth muscle. The lack of net effect on activation of intramural cholinergic neurons, despite the enhanced sensitivity to cholinergic agonists, may indicate reduction in acetylcholine release in lead-treated tissue. (Supported by NIEHS Grant 02665.)

350 INFLUENCE OF ORGANIC SOLVENTS ON THE ANTICOAGULANT RESPONSE TO WARFARIN IN RATS. S. Chakrabarti. Dép. Médecine du travail et hygiène du milieu, Fac. méd., Univ. Montréal, P.Q., Canada

Exposure to styrene (S), trichloroethylene (TCE) and carbon tetrachloride (CT) is known to produce hepatotoxic effects in animals and humans. Warfarin (W), the coumarin anticoagulant, is mostly eliminated by hepatic biotransformation and the site of its anticoagulant action is located in the liver. Therefore, the effects of above solvents on the anticoagulant response to W were studied in male Sprague-Dawley rats. Groups of rats were given i.p. injections of either S (0.6 and 1.2 g/kg) or TCE (5.6 and 11.1 mmole/kg) or CT (1 mmole/kg) in corn oil 24 h prior to or simultaneously with W (1 mg/kg, s.c.) and the animals were sacrificed 24 h after W. Doses of solvents used in this study showed hepatotoxic effects as verified by significant increases in serum transaminases response. A significant increase in prothrombin time (P.T.) was seen when W was treated simultaneously with S or TCE at any dose level, but not so with CT. An increase in the P.T. of W was also noticed in the groups pretreated with the highest dose of S or TCE and with CT group. Increase in serum transaminases activities due to each solvent was not further increased due to W. Solvents alone had no effect on the P.T. In-vivo metabolism of S or TCE was not modified by W. So, acute exposure to organic solvents may lead to enhanced anticoagulant response to W. (Supported in part by IRSST, Québec).

The Toxicologist Vol 5 #1 3/85

351 SERUM BRODIFACOUM CONCENTRATIONS AND COAGULOPATHIC EFFECTS IN ANTICOAGULANT POISONED DOGS TREATED WITH VITAMIN K_1. M.J. Murphy, A.C. Ray, B. Woody and J.C. Reagor. Texas Veterinary Medical Diagnostic Laboratory, College Station, Texas.

In order to correlate coagulopathy with respect to serum brodifacoum (BF) concentrations, four mixed-breed dogs were given a divided LD_{50} dose (1 mg/kg) over a three-day period (0.33 mg/kg/day p.o.). Vitamin K_1 therapy (0.8 mg/kg, TID for 5 days) was initiated at the onset of life-threatening elevations of clotting parameters. Coagulopathy was monitored using packed cell volume, total protein, activated clotting time (ACT), prothrombin time (PT), and partial thromboplastin time. Following ether and ether:acetonitrile extractions of serum, BF concentrations were determined using reverse phase chromatography with U.V. (254 nm, 313 nm) and fluorescent (λ_{exit} 313 nm, λ_{emiss} 375 nm) detection. Recovery from spiked samples was 75% in the 1 ug/ml range and 65% in the 25 ng/ml range. The lower limit of detection was 2 ng/ml. Serum concentrations peaked at 1.15 ug/ml on day 4, following inital exposure. Significant coagulopathy (ACT, 190; PT, 61) was apparent by days 8-10. BF concentrations at the time of initiation of therapy (day 10) were 37-83 ng/ml. Clotting factors returned to normal within 24 hours of Vitamin K_1 therapy. Serum concentrations below 12 ng/ml (after day 15) caused no measurable coagulopathic effect, following cessation of therapy. Elimination of BF followed a classical exponential decay with a distributive phase t 1/2 of 1.4 days and an elimination phase t 1/2 of (8) days.

4-6 days

352 SPECIES COMPARISON OF BONE MARROW PERTURBATIONS IN MICE AND RATS EXPOSED TO ETHYLENE OXIDE. S. Lock, R.E. Hand, Jr. & F. Stenglein, Biol. Div. Oak Ridge National Laboratory, Oak Ridge, TN

Previous studies have shown that exposure of mice (C57B/6J) to 255 ppm of ethylene oxide (ETO) causes an initial loss of granulocytic elements from the bone marrow followed by replacement and hyperproliferation with an associated deficit in the lymphocyte population. In the first of this series of experiments male F344 rats and male BALB/c mice were exposed to 255 ppm ETO for 5 days/week for 2 weeks. Body and spleen weights were recorded for all animals. Bone marrow was counted and aliquots were stained with propidium iodide (PI) for flow cytometric (Ortho 50H) analysis. Forward and 90° scatter parameters allowed quantification of the granulocyte and lymphocyte populations. Cell cycle information was obtained from PI fluorescene histograms. In mice total bone marrow cellularity, lymphocyte and granulocyte populations were depressed by 29%, 58% and 30% respectively following exposure to ETO. Cells in the G1 phase were most severely affected. Conversely rats exposed to ETO had slight increases in total marrow cellularity with both the granulocytic and lymphocytic populations contributing to this increase. It is tentatively concluded that the hematopoietic system of the mouse is more sensitive to ETO than that of the rat. (Research sponsored by OHER, USDOE under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems Inc.)

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The Toxicologist Vol 9 #1 / 2/89 #782

THE EFFECTS OF PHENOBARBITAL INDUCTION ON THE TOXICITY OF BRODIFACOUM IN THE CANINE. M J Murphy, A C Reagor, E M Bailey. Texas Veterinary Medical Diagnostic Laboratory, College Station, TX. Sponsor: A C Ray

A reliable technique for extraction of brodifacoum (BDF) from serum using ether and ether:acetonitrile (1:1) was developed. Two HPLC systems (A: 1.5% Acetic acid, pH 4.5:acetonitrile (1:2)with 1% dibutylamine and B: 0.2 M Tris, pH 7.5 acetonitrile (1:3)) were utilized to optimize sensitivity with simultaneous ultraviolet (254 nm & 313 nm) and fluorescent (313 nm excitation with 375 nm emission) detection. The limit of detection is approximately 10 ng/ml from serum with a recovery of 75±4%.

Dogs (N=5) were induced with 35 mg/kg/day phenobarbital. Induction was demonstrated using antipyrine kinetics [Mean Residence Time: Non-Induced = 117±21; Induced = 65±22 - significant @ P<0.05]. Coagulation parameters were unchanged (Activated Coagulation Time) or prolonged (Prothrombin and Proconvertin Time), and BDF kinetics were unchanged [Area Under the Curve: Non-Induced = 750,000±440,000, Induced = 680,000±250,000] [Mean Residence Time: Non-Induced = 3,000±2,100, Induced = 3,800±900] following induction. Thus, no therapeutic benefit from phenobarbital induction to dogs poisoned with BDF was demonstrated by coagulation status or brodifacoum kinetics.

1. Name and address of first (presenting) author:
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15